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The Course of Etoposide-induced Apoptosis from Damage to DNA and p53 Activation to Mitochondrial Release of Cytochrome *c**

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Natalie O. Karpinich‡§, Marco Tafani‡¶, Ronald J. Rothman‡, Matteo A. Russo¶, and John L. Farber‡‖

From the ‡Department of Pathology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the ¶Department of Experimental Medicine and Pathology, La Sapienza University, Rome 00161, Italy

Treatment of L929 fibroblasts by the topoisomerase II inhibitor etoposide killed 50% of the cells within 72 h. The cell killing was preceded by the release of cytochrome *c* from the mitochondria. Simultaneous treatment of the cells with wortmannin, cycloheximide, furosemide, cyclosporin A, or decylubiquinone prevented the release of cytochrome *c* and significantly reduced the loss of viability. Etoposide caused the phosphorylation of p53 within 6 h, an effect prevented by wortmannin, an inhibitor of DNA-dependent protein kinase (DNA-PK). The activation of p53 by etoposide resulted in the up-regulation of the pro-apoptotic protein Bax, a result that was prevented by the protein synthesis inhibitor cycloheximide. The increase in the content of Bax was followed by the translocation of this protein from the cytosol to the mitochondria, an event that was inhibited by furosemide, a chloride channel inhibitor. Stably transfected L929 fibroblasts that overexpress Akt were resistant to etoposide and did not translocate Bax to the mitochondria or release cytochrome *c*. Bax levels in these transfected cells were comparable with the wild-type cells. The release of cytochrome *c* upon translocation of Bax has been attributed to induction of the mitochondrial permeability transition (MPT). Cyclosporin A and decylubiquinone, inhibitors of MPT, prevented the release of cytochrome *c* without affecting Bax translocation. These data define a sequence of biochemical events that mediates the apoptosis induced by etoposide. This cascade proceeds by coupling DNA damage to p53 phosphorylation through the action of DNA-PK. The activation of p53 increases Bax synthesis. The translocation of Bax to the mitochondria induces the MPT, the event that releases cytochrome *c* and culminates in the death of the cells.

Apoptosis is a process that removes unwanted or damaged cells. The biochemical events that mediate apoptotic cell death are generally initiated in one of two ways. In the first instance, death signals are generated at the cell surface. Activation of such cell surface proteins as the tumor necrosis factor-α or Fas receptors initiate an apoptotic cascade. Alternatively, the deprivation of many trophic growth factors that act through an interaction with a plasma membrane receptor can similarly result in apoptotic cell death. Our current understanding of the events that follow activation of either the tumor necrosis factor-α or Fas receptor envisions an initial premitochondrial phase that involves the Bcl-2 family of pro- and anti-apoptotic proteins and that may or may not require the participation of caspases (1). The mitochondrial phase that follows eventuates in the release of cytochrome *c* and the consequent activation of caspases, enzymes in which action leads to the variety of phenotypic alterations characteristic of apoptotic cell death. The apoptosis consequent to growth factor deprivation is also held to involve an initial phase mediated by the Bcl-2 family of proteins that again results in cytochrome *c* release from the mitochondria followed by a caspase-mediated effector phase (2, 3). Signals that result in apoptotic cell death are also generated from within the cell. Staurosporine and taxol are two well known examples of chemicals that induce apoptosis as a result of an interaction with an intracellular target. In most cases, however, the specific target and the earliest events that ensue upon the interaction with the inducing chemical are poorly understood.

The best known intracellular target for the induction of apoptosis is, of course, DNA. Physical and chemical agents can damage DNA in a variety of ways and with distinct functional consequences, both immediate and delayed. A number of effects on the integrity of the DNA result in the induction of apoptosis, a response that removes cells that can no longer replicate or that have potentially mutagenic damage. The details as to the mechanism whereby the cell recognizes lesions in the DNA that are not readily repairable and then sets in motion events that result in apoptotic cell death are the subject of considerable research efforts. The most dominant current paradigm places p53 at the center of a process that couples DNA damage to the transcriptional regulation of much of the same pro- and anti-apoptotic machinery that is activated by signals originating from the cell surface.

The topoisomerase II inhibitor etoposide is an antineoplastic drug that has been widely used to couple DNA damage to apoptosis (4). Topoisomerase II is a nuclear enzyme that functions during both DNA replication and transcription (5). Topoisomerase II prevents "knots" from forming in DNA by allowing the passage of an intact segment of the helical DNA through a transient double strand break (6). Topoisomerase II inhibitors such as etoposide stabilize the complex formed by topoisomerase II and the 5′-cleaved ends of the DNA, thus forming stable (nonrepairable) protein-linked DNA double strand breaks (6). Cells are apparently able to recognize such DNA damage and, in turn, to eliminate the injured cells by apoptosis.

A substantial literature details many specific biochemical

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‖ To whom correspondence should be addressed: Dept. of Pathology, Thomas Jefferson University, Rm. 208, Jefferson Alumni Hall, Philadelphia, PA 19107. Tel.: 215-503-5066; Fax: 215-923-2218; E-mail: john.farber@mail.tju.edu.

events that occur upon induction by etoposide in a variety of cell types of the apoptotic cascade. Importantly, many of the same steps that are proposed to be central to the mediation of apoptotic cell death in other models have also been reported to occur upon treatment with etoposide. In most cases, these reports deal with a relatively limited portion of a clearly multistep process. Accordingly, how these individual events are coupled to more proximal and distal ones is not fully understood. In the present study, we document a number of distinct manipulations that prevent the apoptotic death of L929 fibroblasts in response to treatment with etoposide. In turn, we use the mechanism of action of these manipulations to detail a sequence that proceeds from the damage to DNA, through p53 to the release of cytochrome *c* from the mitochondria, and that eventuates in the apoptotic death of the cell.

MATERIALS AND METHODS

Cell Culturing Conditions—L929 mouse fibroblasts (ATCC-CCL-1, American Type Culture Collection, Manassas, VA) were maintained in 25-cm^2 polystyrene flasks (Corning Costar Corp., Oneota, NY) with 5 ml of Dulbecco's modified Eagle's medium (DMEM,[1] high glucose, without pyruvate; Mediatech) containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, under an atmosphere of 95% air, 5%CO_2. For the experiments, the cells were trypsinized and plated at a density of 60,000 cells/cm^2. After overnight incubation, the cells were washed twice with phosphate-buffered saline (PBS) and placed in DMEM without serum.

Generation of Akt-overexpressing Stable Transfectants—L929 cells were plated in 24-well plates. After an overnight incubation, the cells were washed in PBS. Transfections were performed using LipofectAMINE-Plus (Invitrogen) according to the manufacturer's protocol. The cells were transfected with 0.5 μg of pCDNA-Akt (7) (generously provided by Dr. Morris J. Birnbaum and Dr. Randall N. Pittman, Howard Hughes Medical Institute, University of Pennsylvania). After 4 h the cells were washed twice with PBS and placed in complete DMEM. After 48 h of further incubation, the cells were washed again with PBS and trypsinized. Cells from 4 wells were plated in 75-cm^2 polystyrene flasks in complete DMEM supplemented with 600 μg/ml G418 (Invitrogen). Stable transfectants were generated and cultured in 25-cm^2 polystyrene flasks. The overexpression of HA-tagged Akt was confirmed by Western blot analysis. Briefly, 5×10^5 cells were pelleted at 700 $\times$ *g* (5 min at 4 °C) and resuspended in 100 μl of cell lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Sigma)). Protein (20 μg) was separated on a 10% SDS-polyacrylamide electrophoresis gel. The gel was electroblotted onto a nitrocellulose membrane and probed with an anti-HA rabbit polyclonal antibody (Y-11, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:4000. The secondary goat anti-rabbit horseradish peroxidase-labeled antibody (1:20,000) was visualized by enhanced chemiluminescence (PerkinElmer Life Sciences).

Treatments—In all experiments etoposide (ETO, Sigma) was dissolved in Me_2SO and added to a final concentration of 10 μM. Where indicated the cells were pretreated for 30 min with the following compounds. Wortmannin (Biomol, Plymouth Meeting, PA) was dissolved in Me_2SO and added to the cells to give a final concentration of 200 nM. Cycloheximide (Sigma) was dissolved in PBS and added to a final concentration of 1 μM. Furosemide (Sigma) was dissolved in Me_2SO to give a final concentration of 2 mM. Cyclosporin A and aristolochic acid (both from Biomol) were dissolved in Me_2SO and added to a final concentration of 5 and 50 μM, respectively. Decylubiquinone (DUBQ, Biomol) was dissolved in Me_2SO and added to the cells at a final concentration of 5 μM. Control experiments showed that Me_2SO had no effect on any of the parameters measured.

Measurement of Cell Viability—Cell viability was determined by trypan blue exclusion. After treating the cells, 10 μl of 0.5% trypan blue solution was added directly into each well containing 500 μl of medium. Both viable and nonviable cells were counted for each data point in a total of eight microscopic fields.

[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; DNA-PK, DNA-dependent protein kinase; MPT, mitochondrial permeability transition; PBS, phosphate-buffered saline; ETO, etoposide; Me_2SO, dimethyl sulfoxide; DUBQ, decylubiquinone; COX IV, cytochrome oxidase subunit IV; PI3-kinase, phosphoinositide 3-kinase; HSP70, heat shock protein 70; CyA, cyclosporin A.

TABLE I
Time course of the killing of L929 fibroblasts by ETO

L929 fibroblasts were treated with 10 μM ETO or pretreated for 30 min with each of the compounds used below followed by the addition of ETO. The viability of cells was determined by trypan blue exclusion at the times indicated. The results are the mean ± S.D. of three separate experiments.

Treatment	% Dead cells		
	24 h	48 h	72 h
Control	0	3.9 ± 3.0	7.6 ± 3.6
Etoposide	9.8 ± 2.7	30.5 ± 2.4	48.1 ± 2.4
ETO + wortmannin	1.2 ± 0.2	8.4 ± 1.7	16.4 ± 2.8
ETO + cycloheximide	0.8 ± 0.8	10.0 ± 4.2	15.9 ± 3.1
ETO + furosemide	1.8 ± 0.9	10.7 ± 2.4	14.5 ± 2.5
ETO + DUBQ	3.4 ± 1.5	20.6 ± 5.2	20.4 ± 1.1

Isolation of Cytosol and Mitochondrial Fractions—Cells were plated in 75-cm^2 flasks at a density of 4.5×10^6 cells/flask. After treatment, the cells were scraped off the flasks followed by centrifugation at 1000 $\times$ *g* for 10 min at 4 °C. The cell pellets were resuspended and washed once in PBS followed by an additional centrifugation at 700 $\times$ *g* for 10 min at 4 °C. The cell pellets were resuspended in 1 ml of Buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). Cells were lysed by 15 passages through a 26-gauge needle, and homogenates were centrifuged at 1000 $\times$ *g* for 5 min at 4 °C. The supernatants were then centrifuged at 10,000 $\times$ *g* for 15 min at 4 °C. The resulting mitochondrial pellets were resuspended in 50 μl of cold cell lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Sigma)). The supernatant from the final centrifugation was used for the preparation of concentrated cytosol. The supernatant was centrifuged at 14,000 $\times$ *g* for 35 min at room temperature using Microcon centrifugal filter devices (Millipore, Bedford, MA) with molecular weight exclusion at 10,000.

Western Blot Analysis of Cytochrome c Release and Bax Translocation—The mitochondrial and cytosolic fractions were used for detection of cytochrome *c* and Bax. These fractions (30 μg of cytosol and 15 μg of mitochondria) were separated on 15% SDS-polyacrylamide electrophoresis gels with an equal amount of protein loaded onto each lane as determined by bicinchoninic acid assay. Kaleidoscope prestained standards (Bio-Rad, Hercules, CA) were used to determine molecular weights. The gels were then electroblotted onto nitrocellulose membranes. Cytochrome *c* was detected by a mouse monoclonal antibody (PharMingen, San Diego, CA) at a dilution of 1:500. Secondary goat anti-mouse horseradish peroxidase-labeled antibody (1:15,000) was detected by enhanced chemiluminescence. Bax (N-20) was detected with a rabbit polyclonal antibody at a dilution of 1:500 (Santa Cruz Biotechnology). Secondary goat anti-rabbit horseradish peroxidase-labeled antibody (1:15,000) was visualized by enhanced chemiluminescence. As a control for the purity of cytosolic and mitochondrial fractions, an anti-bovine cytochrome oxidase subunit IV antibody (Molecular Probes, Eugene, OR) and a mouse monoclonal mitochondrial heat shock protein 70 antibody (Affinity BioReagents, Inc., Golden, CO) were used at a dilution of 1:500.

Determination of Total p53, Phospho-p53, and Total Bax, c-Myc, and Bcl-2—Cells were plated in 25-cm^2 flasks in complete DMEM. After an overnight incubation, the cells were washed with PBS and placed in serum-free DMEM for 16 h. The following day, the cells were treated and then harvested by scraping, and the cells were then centrifuged at 1000 $\times$ *g* for 10 min at 4 °C. The cell pellets were resuspended in 1 ml of PBS and centrifuged again at 700 $\times$ *g* for 10 min. The resulting pellet was resuspended in 50 μl of cell lysis buffer and kept on ice for 30 min. Proteins were separated on a 10% (p53, phospho-p53, c-Myc) or 15% (total Bax, Bcl-2) SDS-polyacrylamide electrophoresis gels and then electroblotted onto nitrocellulose membranes. Total p53 was detected by mouse monoclonal antibody at a dilution of 1:250 (PharMingen). Phospho-p53 (Ser-15) was detected by a rabbit polyclonal antibody used at a dilution of 1:1000 (New England BioLabs, Beverly, MA). Bax (B-9) was detected by a mouse monoclonal antibody at a dilution of 1:1000 (Santa Cruz Biotechnology). The c-Myc protein was detected using a rabbit polyclonal antibody at a dilution of 1:250 (Santa Cruz Biotechnology). Bcl-2 (C-2) was detected using a mouse monoclonal antibody at a dilution of 1:500 (Santa Cruz Biotechnology). The secondary antibodies used for total p53, phospho p53, c-Myc, Bcl-2, and Bax were goat anti-mouse (1:15,000), goat anti-rabbit (1:25,000), and goat anti-mouse

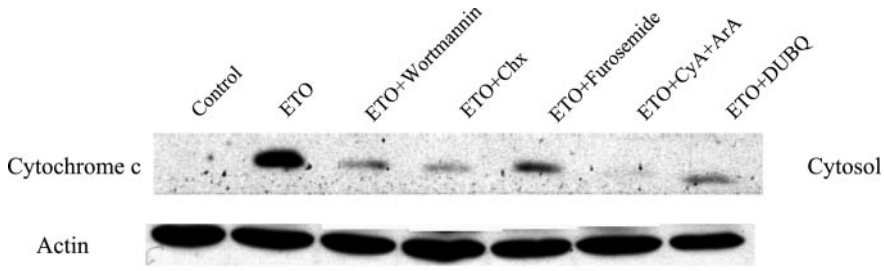

FIG. 1. **Release of cytochrome *c* into the cytosol following etoposide treatment.** L929 fibroblasts (4.5×10^6) were treated with 10 μM ETO or pretreated for 30 min with 200 nM wortmannin, 1 μM cycloheximide (*Chx*), 2 mM furosemide, 5 μM CyA and 50 μM aristolochic acid (*ArA*), or 5 μM DUBQ followed by the addition of ETO. After 24 h, cytosolic fractions were prepared, and the release of cytochrome *c* was detected by Western blotting. Actin was used as a control for equal loading.

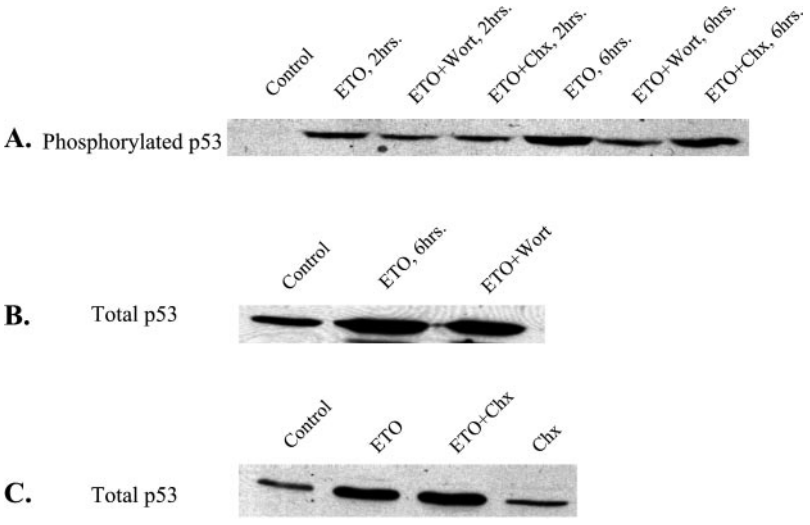

FIG. 2. **Wortmannin prevents the phosphorylation of p53.** *A*, cells (1.3×10^6) were treated with 10 μM ETO or pretreated for 30 min with 200 nM wortmannin (*Wort*) or 1 μM cycloheximide (*Chx*) followed by the addition of ETO. After 6 h, the levels of p53 phosphorylation were determined by Western blot analysis. *B* and *C*, cells were treated as described in *A*. After 6 h, the levels of total p53 were determined by Western blot analysis.

(1:15,000) horseradish peroxidase-labeled antibody, respectively, and the results were visualized by enhanced chemiluminescence.

RESULTS

Prevention of the Release of Cytochrome c in Fibroblasts Treated with Etoposide—The release of cytochrome *c* from the mitochondrial intermembraneous space into the cytosol is a prominent downstream manifestation of the evolution of apoptotic cell death. The killing of L929 fibroblasts with etoposide is accompanied by a similar release of cytochrome *c*. Twenty-four hours after treating L929 fibroblasts with 10 μM ETO, 90% of the cells are still viable (Table I). Fig. 1 shows, however, that after 24 h cytochrome *c* is present in the cytosol. The content of actin in the same cytosolic fractions did not differ (Fig. 1), and the mitochondrial marker COX IV was not present (data not shown). Thus, the presence of cytochrome *c* in the cytosol with ETO represents its release from the mitochondria.

Fig. 1 also shows that the treatment of L929 fibroblasts with ETO in the presence of one of five different chemicals (wortmannin, cycloheximide, furosemide, cyclosporin A, or decylubiquinone) prevents the release of cytochrome *c* into the cytosol. Again, the effect of these agents cannot be attributed to changes in the purity of the cytosolic fractions (Fig. 1).

Wortmannin Prevents the Phosphorylation of p53 Induced by Etoposide—The inhibition of topoisomerase II by etoposide results in the accumulation of double strand breaks in DNA. Such damage to DNA is recognized by and results in the activation of DNA-dependent protein kinase (8). DNA-PK is a member of the PI3-kinase family that phosphorylates and thereby activates p53 (8, 9). Wortmannin is an inhibitor of the catalytic subunit of the PI3-kinase family of enzymes, including DNA-PK (10). Fig. 2*A* shows that 200 nM wortmannin inhibits the phosphorylation of p53 that is evident 6 h after treatment of L929 fibroblasts with ETO. The total content of p53 was unchanged in the whole cell lysates used to determine this effect of wortmannin (Fig. 2*B*).

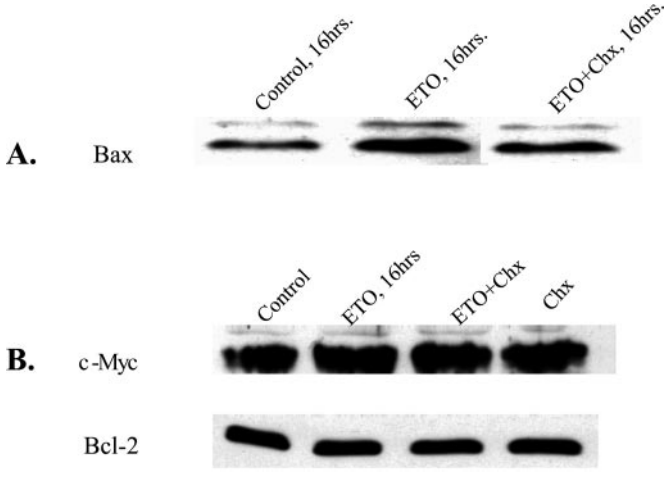

FIG. 3. **Cycloheximide reduces the expression of Bax after ETO treatment.** *A*, L929 fibroblasts (1.3×10^6) were treated with 10 μM ETO or pretreated for 30 min with 1 μM cycloheximide (*Chx*) followed by the addition of ETO. Cells were lysed after 16 h, and total Bax (B-9) levels were determined by Western blot analysis. *B*, total lysates were obtained for cells treated with 10 μM ETO or cells pretreated for 30 min with 1 μM cycloheximide followed by the addition of ETO. After 16 h, levels of c-Myc and Bcl-2 were determined by Western blot analysis.

The reduced phosphorylation of p53 was reflected, in turn, by a decrease with wortmannin in the extent of cell killing by ETO. Table I details the loss of viability with ETO over a 72-h time course. At most, 10% of the cells die within 24 h, whereas 30% are dead after 48 h and almost 50% within 72 h. In the presence of 200 nM wortmannin, the extent of cell killing was significantly reduced throughout this same period. After 72 h, wortmannin reduced 3-fold the extent of cell killing produced by ETO.

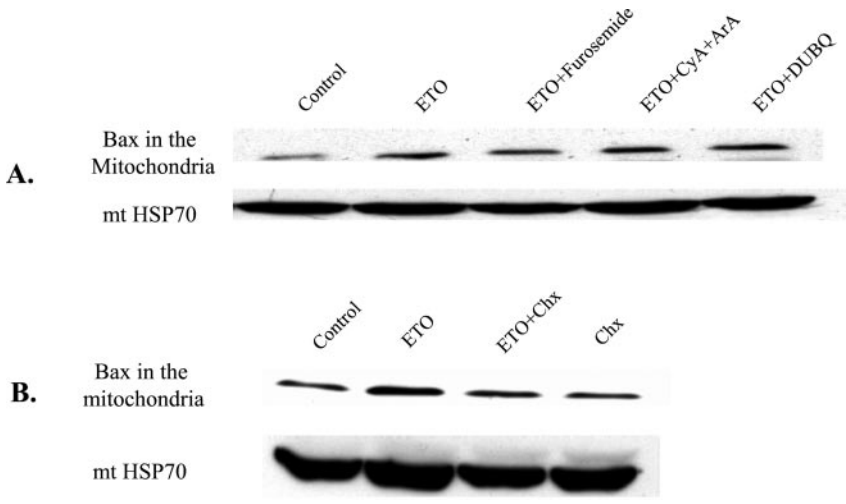

FIG. 4. **Furosemide prevents the translocation of Bax to the mitochondria in cells treated with ETO.** *A*, L929 fibroblasts (4.5×10^6) were treated with 10 μM ETO or pretreated for 30 min with 2 mM furosemide, 5 μM CyA and 50 μM aristolochic acid (*ArA*), or 5 μM DUBQ followed by the addition of ETO. After 18 h, the content of translocated Bax was determined in a mitochondrial subcellular fraction by Western blot. Mitochondrial HSP70 was used as a control for purity of the fraction and to ensure equal loading of the protein. *B*, cells (4.5×10^6) were treated with 10 μM ETO or pretreated for 30 min with 1 μM cycloheximide followed by the addition of ETO. A Western blot of the mitochondrial subcellular fraction showed the content of translocated Bax after 18 h. Again, mitochondrial HSP70 was used as a control for the samples.

FIG. 5. **Akt overexpression in L929 fibroblasts.** L929 cells (clones *1*, *7*, and *8*) were stably transfected with an Akt expression vector. The overexpression of Akt was determined by Western blot analysis. *WT*, wild type.

TABLE II
Overexpression of Akt protects against the cytotoxicity of ETO

L929 wild type (WT) and Akt-overexpressing cells were treated with 10 μM ETO. The viability of cells was determined by trypan blue exclusion. The results are the mean ± S.D. of three separate experiments.

Treatment	% Dead cells at 72 h
WT control	7.6 ± 3.6
WT ETO	48.1 ± 2.4
Akt8 + ETO	18

Cycloheximide Prevents the Expression of Bax Induced by Etoposide—Bax is a proapoptotic protein whose expression is regulated by p53 (11). In turn, Bax acts on the mitochondria to cause the release of cytochrome *c* (12). Fig. 3*A* shows that there is a significant increase in the total Bax content of L929 fibroblasts 16 h after treatment with ETO. The protein synthesis inhibitor cycloheximide reduced this increased expression of Bax (Fig. 3*A*). In the presence of cycloheximide, phosphorylation of p53 was still readily detectable (Fig. 2*A*), a result that contrasts with the action of wortmannin (Fig. 2*A*). The content of p53 in a whole cell lysate was not different in cells treated with etoposide alone or etoposide plus cycloheximide (Fig. 2*C*). Cycloheximide did not reduce the content of two other proteins (c-Myc and Bcl-2) that could potentially play a role in the cell killing by etoposide (Fig. 3*B*).

The inhibition of Bax expression by cycloheximide was also accompanied by protection against cell killing by ETO. To an extent very similar to the effect of wortmannin, cycloheximide reduced the loss of viability seen with ETO (Table I). Whereas over 50% of the fibroblasts died after 72 h with ETO alone, 85% of the cells were still viable in the presence of cycloheximide.

Furosemide Inhibits the Translocation of Bax to the Mitochondria—Bax moves from the cytosol to the mitochondria under conditions that induce cell death by apoptosis (13). Although the mechanism that mediates this translocation of Bax is not fully understood, evidence suggests that it is a consequence of a conformational alteration in the protein as a result of changes in the ionic composition of the cytosolic milieu (14–16). Furosemide is a chloride channel inhibitor that changes the pH and/or ionic strength within the cell (17).

In addition to increasing the total content of Bax, ETO causes the translocation of Bax to the mitochondria, as shown by the increased content of Bax in the mitochondria isolated from L929 fibroblasts treated with ETO (Fig. 4*A*). Despite this increase in Bax in the mitochondria from treated as opposed to control cells, the content of HSP70 in the same samples remained unchanged (Fig. 4*A*), a result indicating that changes in the purity of the preparations cannot account for the difference. The translocation of Bax was prevented by 2 mM furosemide (Fig. 4*A*). Importantly, furosemide did not prevent the increase in total Bax content (data not shown). Consistent with the inhibition of Bax synthesis, cycloheximide also prevented the increase in the mitochondrial content of Bax that occurs with etoposide (Fig. 4*B*).

The inhibition of Bax translocation by furosemide was again accompanied by protection from cell killing by ETO. To an extent very similar to the effects of wortmannin and cycloheximide, furosemide reduced the loss of viability seen with ETO (Table I). Whereas over 50% of the fibroblasts died after 72 h with ETO alone, 85% of the cells were still viable in the presence of furosemide.

Decylubiquinone Prevents Cell Killing by Etoposide—Bax can act on the mitochondria to induce the mitochondrial permeability transition (18). Decylubiquinone and cyclosporin A are known inhibitors of the MPT (18, 19). Fig. 1 shows that decylubiquinone and cyclosporin A prevent the release of cytochrome *c* into the cytosol after 24 h of ETO treatment. Table I shows that after 3 days, 50% of the ETO-treated cells were killed, whereas those treated also with DUBQ had only a 20% loss of viability. The effect of CyA was more complex. CyA prevented the release of cytochrome *c* as determined 24 h after treatment with ETO (Fig. 1). At this time, there was very little loss of viability (Table I). Dead cells accumulated over the next 48 h, and CyA was not able to prevent the loss of viability over this longer time course. The action of CyA as an inhibitor of the MPT is known to be self-limited (20, 21), and its failure to prevent the toxicity of ETO at 48 and 72 h is likely a consequence of this fact.

Overexpression of Akt Protects against Cell Killing by Etoposide—The cytosolic protein kinase Akt is implicated in an anti-apoptotic signaling pathway (22, 23). Akt phosphorylates the pro-apoptotic protein Bad, thereby making it unable to bind the anti-apoptotic proteins Bcl-X or Bcl-2 (24, 25). Bcl-X and Bcl-2 are then free to bind Bax and prevent its translocation to the mitochondria. To further study the role of Bax in ETO-induced apoptosis, stably transfected L929 fibroblasts that overexpress

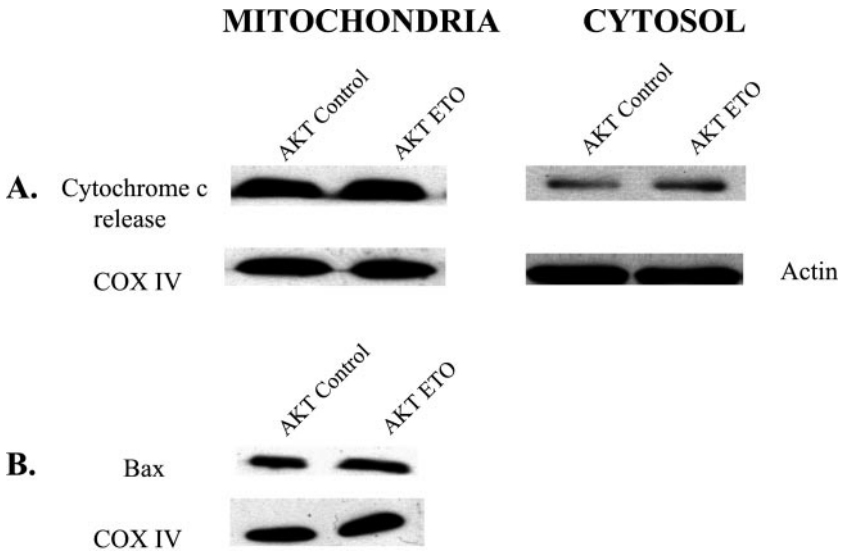

FIG. 6. **Akt overexpression prevents both the release of cytochrome *c* from the mitochondria and Bax translocation to the mitochondria.** *A*, L929 fibroblasts overexpressing Akt were treated with 10 μM ETO for 24 h. Mitochondrial and cytosolic fractions were isolated and release of cytochrome *c* was detected by Western blot analysis. COX IV and actin were used as controls to ensure the equal loading of the mitochondrial and cytosolic fractions, respectively. *B*, clones overexpressing Akt were treated with 10 μM ETO for 18 h. Mitochondrial fractions were isolated, and translocation of Bax was detected by Western blot analysis. COX IV was used to control for equal protein loading of the gel.

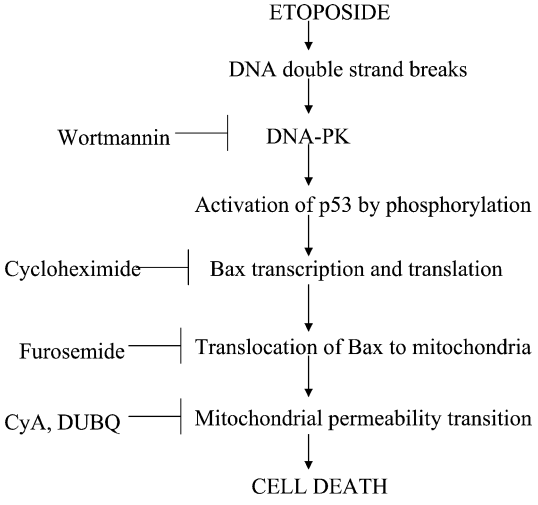

FIG. 7. **Mechanism of ETO-induced apoptosis in L929 fibroblasts.**

Akt were generated (Fig. 5). The clones that overexpress Akt are resistant to ETO. Table II shows that after 3 days, Akt-overexpressing clones reduced cell killing by ETO by almost 3-fold. Both the translocation of Bax to the mitochondria and the release of cytochrome *c* into the cytosol were prevented by the overexpression of Akt (Fig. 6). Importantly, Akt overexpression did not prevent p53 phosphorylation or an increase in total Bax content (data not shown).

DISCUSSION

We have identified five distinct chemicals and one genetic manipulation that significantly reduce cell killing by etoposide. In addition, each of these manipulations prevented a characteristic phenomenon in the evolution of the apoptotic phenotype, namely the release of cytochrome *c* from the mitochondria. Five distinct biochemical events were identified as the likely respective targets of the action of each of these manipulations. In turn, these events constitute a sequence that proceeds from DNA damage through p53 phosphorylation to Bax up-regulation, its subsequent translocation to the mitochondria with the resultant release of cytochrome *c* into the cytosol, and ultimately, cell death.

The topoisomerase II inhibitor etoposide causes an accumulation of double strand breaks within the nuclei of cells. These breaks are recognized by the multiprotein complex DNA-dependent protein kinase, or more specifically, the heterodimer of Ku subunits that bind to the double-stranded DNA ends (26, 27). By binding to DNA, Ku recruits and activates the catalytic subunit (26). The catalytic subunit of DNA-PK is a member of the PI3-kinase family (9, 28). The PI3-kinase inhibitor wortmannin significantly reduced the extent of cell killing (Table I). By preventing the DNA-PK catalytic subunit from recognizing the double strand breaks induced by ETO, all downstream manifestations of apoptotic cell death including phosphorylation of p53 are inhibited, thus maintaining the viability of the cells. In the apoptotic cascade, the activation of DNA-PK is pivotal because it provides the necessary link between recognition of DNA damage and the subsequent downstream signaling events.

The tumor suppressor protein p53 is a regulator of cell cycle progression and mediator of apoptosis in many cell lines. Activation of p53 occurs through phosphorylation (29). In particular, the Ser-15 in p53 of humans (which corresponds to Ser-18 of mouse) is a substrate for phosphorylation by DNA-PK (30–32). Phosphorylation of p53 occurred after treatment of L929 fibroblasts with 10 μM ETO (Fig. 2*A*). By inhibiting DNA-PK (26), wortmannin prevented the phosphorylation of p53, an effect that, in turn, inhibited the subsequent downstream events that culminate in cell death.

It is conceivable that the protection afforded by wortmannin reflects an inhibition of a member of the PI3-kinase family other than DNA-PK. In this regard, it is noteworthy, at least, that the best known member of this family, the PI3-kinase that phosphorylates Akt in the cytosol, is anti-apoptotic (24). That is, this PI3-kinase functions in a pathway that prevents apoptosis. Accordingly, inhibition of this kinase by wortmannin potentiates apoptotic cell death (33). Thus, the protective effect of wortmannin against cell killing by etoposide in the present study must reflect inhibition of a pro-apoptotic PI3-kinase rather than inhibition of an anti-apoptotic PI3-kinase. In turn, the demonstration here that wortmannin prevents the phosphorylation of p53 is clearly consistent with inhibition of a pro-apoptotic event, namely inhibition of DNA-PK.

Phosphorylation of p53 results in the up-regulation of proteins implicated in cell cycle control and apoptosis (34). In particular, Bax is a pro-apoptotic protein that is transcriptionally regulated by p53 (11, 35). Treatment of L929 fibroblasts with etoposide increased the content of Bax (Fig. 3*A*). By inhibiting protein synthesis and thus the increase in the content of Bax, cycloheximide protected against cell killing by ETO (Table I).

The pro-apoptotic action of Bax is believed to be mediated by

its interaction with the mitochondria, in particular, its insertion into the outer mitochondrial membrane (36). Whereas overexpression of Bax leads to mitochondrial permeabilization and cell death (37), evidence suggests that mechanisms in addition to an increase in the content of the protein are necessary for Bax to translocate from the cytosol to the mitochondria (13). It is suspected that a conformational change in Bax results in the exposure of its N-terminal domain, an event that may free the hydrophobic C-terminal membrane-anchoring domain (14–16). Several mechanisms have been proposed to account for such a Bax conformational change, including an alteration in intracellular pH (an alkalinization of the cytosol) and/or an interaction with the pro-apoptotic protein Bid (38). In this regard, the data presented above suggest that the increase in content of Bax produced by ETO is not sufficient to induce cell killing.

Pretreatment of L929 fibroblasts with the chloride channel inhibitor furosemide reduced the translocation of Bax to the mitochondria (Fig. 4*A*). Consequently, furosemide prevented the release of cytochrome *c* from the mitochondria (Fig. 1) and reduced the extent of cell killing (Table I). Importantly, furosemide did not prevent the increase in the content of Bax. We would argue that, by inhibiting a plasma membrane chloride channel, furosemide alters the ionic strength within the cytosol, an effect that prevents a conformational change in Bax that would otherwise render it susceptible to mitochondrial translocation.

The translocation of Bax to the mitochondria in response to treatment with ETO was also prevented by overexpression of Akt (Fig. 6*B*), a result that was again reflected in the absence of cytochrome *c* release (Fig. 6*A*) and resistance to cell killing (Table II). Importantly, the cellular content of Bax was not affected by the overexpression of Akt (data not shown). Akt is a serine-threonine kinase that phosphorylates the pro-apoptotic protein Bad (24). Upon its phosphorylation, Bad no longer binds to the anti-apoptotic protein Bcl-X, thereby freeing the latter to bind to Bax and to prevent Bax translocation to the mitochondria.

Upon its translocation to the mitochondrion, Bax can cause the release of cytochrome *c* (12, 36). The mechanism by which Bax releases cytochrome *c* is a matter of some current debate (39–41). We have shown that the release of cytochrome *c* by Bax from both isolated mitochondria *in vitro* (18) and from these organelles in the intact cell (37) is a consequence of the opening of the permeability transition pore. In turn, opening of the permeability transition pore can lead to induction of the mitochondrial permeability transition (MPT). Cyclosporin A and decylubiquinone, inhibitors of the MPT, prevented the release of cytochrome *c* in L929 fibroblasts treated with ETO (Fig. 1) and reduced the loss of viability (Table I). However, CyA did not prevent cell killing past 24 h (data not shown), because the protective effects of CyA are transient (20, 21). Whereas CyA is believed to exert its anti-apoptotic effects through binding to cyclophilin D, DUBQ is a potent MPT inhibitor by binding to a ubiquinone binding site that appears to be involved directly in permeability transition pore regulation (19). Unlike CyA, DUBQ was able to prevent cell killing by ETO over a 3-day time course (Table I).

The various manipulations discussed above that modify the response of L929 fibroblasts to etoposide can be summarized by the sequence presented in Fig. 7. ETO induces the accumulation of DNA double strand breaks that are subsequently recognized by DNA-PK. This multiprotein complex then activates p53 through phosphorylation. Upon activation, p53 causes an increase in the transcription of the pro-apoptotic protein Bax. Bax undergoes a conformational change and is able to translocate to the mitochondria. This movement of Bax to the mitochondria induces the MPT, an event that results in the release of cytochrome *c* and culminates with loss of viability of the cells.

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The Course of Etoposide-induced Apoptosis from Damage to DNA and p53 Activation to Mitochondrial Release of Cytochrome *c*

Natalie O. Karpinich, Marco Tafani, Ronald J. Rothman, Matteo A. Russo and John L. Farber

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